Molecular Cloning of cDNA for BRab from the Brain of *Bombyx mori* and Biochemical Properties of BRab Expressed in *Escherichia coli*†

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Received March 27, 1998

From a brain cDNA library of *Bombyx mori*, we cloned cDNA for BRab, which encoded a 202-amino-acid polypeptide sharing 60–80% similarity with rab1 family members. To characterize its biochemical properties, cDNA for BRab was inserted into an expression vector (pGEX2T) and expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein. The recombinant protein was purified to homogeneity with glutathione S-Sepharose. The purified GST-BRab bound [35S]-GTPyS and [3H]-GDP with association constants of 1.5 × 10^5 M^-1 and 0.58 × 10^5 M^-1, respectively. The binding of [35S]-GTPyS was inhibited with GDP and GTP, but with no other nucleotides. The GDP-hydrolysis activity was evaluated to be 5 μ mole/min/mole of BRab. In the presence of 6 mM MgCl2, bound [35S]-GTPyS and [3H]-GDP were exchanged with GTPyS most efficiently. These results suggest that BRab, having a higher affinity for GTP than GDP, converts from the GTP-bound state into the GDP-bound state by intrinsic GTP hydrolysis activity and returns to the GTP-bound state with the exchange of GDP with GTP.

Key words: *Bombyx mori*; small GTP binding protein; cDNA cloning

Small GTP binding proteins regulate many biological functions such as cell proliferation, cell division, and protein translocation.1–3) Among these, rab family proteins (ras-like GTP binding protein) are involved in the regulation of vesicular transport through the endocytic and secretory pathways.4–9) Each rab is thought to act at a particular stage of a vesicular transport. For example rab1 and rab2 function in the transport pathways from the ER to the Golgi complex.10) Rab4 and rab5 are required for the endocytic pathway between the plasma membrane and early endosomes.11) Rab 3A regulates the exocytosis of synaptic vesicles.12)

It is accepted that neuropeptides are synthesized into endoplasmic reticulum (ER), transported to the Golgi complex to form secretory vesicle in neurosecretory cells, and eventually released into the hemolymph in insects. Probably rab family proteins are associated with these steps of neuropeptide translocation.

PTTH (prothoracotrophic hormone) is a critical neuropeptide hormone to control biosynthesis and release of ecdysone from the prothoracic gland, resulting in morphological changes and ecdysis in insects. PTTH is synthesized in the neurosecretory cell body in the brain, and periodically released from axonal terminus in the corpora allata.5,6) In insect brain, neurotransmitters were found to regulate the secretion of neuropeptides such as PTTH. For example, carbachol (acetyl choline agonist) was found to stimulate in vitro release of PTTH via muscarinic acetylcholine receptor from the neurosecretory cells of brain-corpus cardiacum-corpus allatum complex (Br-CC-CA) of *Bombyx mori*.10–12) Rab3A, one of the rab family proteins, functions in secretion of neurotransmitter in vertebrate brain,13,14) however whether the secretion of neuropeptides and neurotransmitters in insect brain is related to rab family protein function is unknown. Therefore, it is important to elucidate the molecular properties of rab family protein in insect brain.

In this study, we isolated a cDNA clone of small GTP binding protein from a brain cDNA library of *B. mori*. The amino acid sequence deduced from its cDNA was similar to that of rab1, which regulates the vesicle transport from the ER to the Golgi complex in vertebrates.4) The cloned cDNA was expressed in *E. coli*, and then the expressed-protein was purified and characterized.

Materials and Methods

**Materials.** The cDNA library of *B. mori* (1–5 day after 4th ecdysis) was kindly provided by Dr. Iwami (Kanazawa University). [3H]-GDP and [35S]-GTPyS were purchased from ICN Biomedicals Inc. GTP, GDP, and GTPyS were from Nakalai Tesque Inc. Quick Prep Micro mRNA Purification kit, glutathione S-Sepharose, and pGEX2T were from Pharmacia LKB. Nitrocellulose membrane filter (025 mm, 0.45 μm) was from Advantec Toyo Roshi Kaisha, Ltd. PEI-cellulose TLC-plate was from Machery-Nagel, Germany. The other chemicals were of the purest grade commercially available.

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† This work was supported in part by a Grant-in-Aid for Research on Priority Areas, No. 08276101 from the Ministry of Education, Science, Sports, and Culture of Japan, and The Kurata Research Grant from The Kurata Foundation.

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Isolation of cDNA Clones. mRNA was prepared from brains of B. mori with Quick Prep Micro mRNA Purification kit (Pharmacia). One μg mRNA was reverse-transcribed with a mixture of avian myeloblastosis virus (AMV) reverse transcriptase and random hexamer primers. PCR was done between a forward primer, 5'-TCAAACTCAGATCTGGGACACCTGCTG-3' and a reverse primer, 5'-ACCCGGCTGTT-CCTCCATGCGACCTTTT-3'. The temperature cycles were 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C. The amplified DNA fragment of 220 bp was recovered. The brain cDNA library was screened with this fragment. Three positive clones were isolated and their nucleotides sequenced by a DNA sequencer, LI-COR 4000L Infrared automated sequencer (Aloka).

Construction of Expression Plasmid and Expression of cDNA for BRab in E. coli. The cDNA fragment containing the entire coding sequence was amplified by PCR with primers containing the EcoRI site. The amplified fragment was digested with EcoRI and introduced into the EcoRI site of an expression vector, pGEX2T, to generate expression plasmid, pGEX-BRab. This cDNA was transformed into E. coli strain DH5α. The DNA sequence of the constructed expression vector was confirmed by DNA sequencing. Ten ml of a overnight culture containing the appropriate plasmid in LB-medium was used as an inoculum for 1 l of LB-medium containing 100 μg/ml ampicillin and 0.1% glucose. Growth continued at 30°C for 3 hours and then the culture was induced with 100 μM isopropyl β-D-(−)-thiogalactopyranoside (IPTG) for 72 hours at 30°C. The cells were collected by centrifugation at 5,000×g for 5 min, and then stored at −80°C.

Purification of GST-BRab. Purification was done at 4°C. The frozen cells were suspended in phosphate-buffered saline (PBS) [140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 (pH 7.3)] and sonicated 3 times for 1 min. The mixture of destroyed cells was centrifuged at 8,000×g for 1 hour. The supernatant was put directly on a glutathione-Sepharose column (2.0×10 cm) equilibrated with PBS. The column was washed with PBS and developed with 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 10 mM glutathione at a flow rate of 1 ml/min. Protein was measured by the method of Lowry et al.14

SDS-PAGE. SDS-PAGE was done by the method of Laemmli,15 using a 4.5% stacking gel and 15% separating gel. Protein in the gel was stained with Coomassie brilliant blue.

GTPβS and GDP Binding Assay. To examine the course of [35S]-GTPβS binding, GST-BRab (2.4 μg) was incubated at 30°C for the indicated period of time in 100 μl of reaction mixture [50 mM Tris-HCl (pH 8.0), 40 μM [35S]-GTPβS, and 10 mM MgCl2]. The reaction was stopped by adding 1 ml of washing buffer (20 mM Tris-HCl (pH 8.0), 25 mM MgCl2, 0.1 M NaCl). The reaction mixture was filtrated through a nitrocellulose membrane filter (φ25 mm, 0.45 μm). The membrane was washed twice with 1 ml of ice-cold washing buffer, dried at 30°C overnight, and the filter-bound radioactivity was counted by a liquid scintillation counter, Aloka, LSC-5100. When the effect of MgCl2 on the binding activity was examined, GST-BRab (2.4 μg) was dialyzed overnight against 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. GTP binding was assayed at 30°C for 120 min in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 2.4 μM GST-BRab, 40 μM [35S]-GTPβS, and various concentrations of MgCl2. GTPβS binding was assayed at 30°C for 120 min in 100 μl of a mixture containing 2.4 μg GST-BRab, 6 mM MgCl2, 50 mM Tris-HCl (pH 8.0) and various concentrations of [35S]-GTPβS. GDP binding was measured this way except for using [3H]-GDP instead of [35S]-GTPβS. Non-specific bindings of [3H]-GDP and [35S]-GTPβS to GST-BRab were measured by adding a 1,000-fold excess of unlabelled GDP and GTPβS, respectively, to the reaction mixture.

Measurement of GTPase Activity. GST-BRab (2.4 μg) was incubated in a 5-μl mixture containing 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 0.1 mM EDTA to remove GDP at 0°C for 5 min, and incubated at 30°C for the indicated period of time in 25 μl of reaction mixture [50 mM Tris-HCl (pH 7.8), 1 mM DTT, 20 μM [3H]-GTP and 6 mM MgCl2 or without MgCl2]. The reaction was stopped by adding 100 μl of 0.5 M EDTA. One μl of GTP-GDP mixed solution (0.1 μmole, each) was mixed with 20 μl of reaction mixture and left for 10 min. Four μl of the mixture was spotted on a PEI-cellulose TLC-plate, which was then developed in 1 M LiCl. After drying, the spots of GTP and GDP were seen under UV illumination. The spot of GDP was excised and its radioactivity was counted. As a control, GST was used instead of GST-BRab. The exact values were calculated by subtracting the values with GST from those with GST-BRab.

Exchange of Bound [35S]-GTPβS and [3H]-GDP with Guanine Nucleotides. [35S]-GTPβS-bound and [3H]-GDP-bound GST-RBabs were made by incubating GST-BRab (2.4 μg) for 120 min at 30°C in a reaction mixture (90 μl) containing 50 mM Tris-HCl (pH 8.0), 6 mM MgCl2, and either 20 μM [35S]-GTPβS or 20 μM [3H]-GDP. The exchanges of [35S]-GTPβS and [3H]-GDP on GST-BRab were started, respectively, by adding a 1,000-fold excess of either unlabeled GTPβS or GDP. After incubation for various periods at 30°C, the non-exchanged [35S]-GTPβS or [3H]-GDP was trapped on a nitrocellulose membrane filter and its radioactivity was measured as described for the [35S]-GTPβS binding assay. Non-specific binding was assayed by incubation of GST-BRab with 20 μM [35S]-GTPβS and 20 mM GTPβS or with 20 μM [3H]-GDP and 20 mM GDP.

Results

Isolation of cDNA Clones

At the primary structure, every rab contains 4 characteristic regions (Fig. 1). These regions are involved in GTPase catalytic sites (I and II) and GTP/GDP binding
sites (III and IV). We synthesized forward and reverse oligonucleotide primers for the PCR within the GTPase-related site (II) and the GTP/GDP binding site (III), respectively, based on rab3 from Drosophila melanogaster. To our knowledge, Rab3 is the only small GTP binding protein the cDNA sequence of which has been described from an insect to date (accession number; M64621). As a result, a 220-bp fragment was amplified by reverse-transcribed (RT)-PCR, cloned, and sequenced. Subsequently, a brain cDNA library of B. mori was screened with this PCR-amplified fragment. Three positive clones were isolated and their DNA sequences were analyzed. Two of these clones had an identical sequence that was similar to the small GTP binding protein, but the other clone had no similarity. As a result, we isolated a complete cDNA clone for Rabrab 952 bp in length (accession number; AF013572). The nucleotide sequence showed an open reading frame coding for 202 amino acids in a protein that is closely related to rab1 family members (Fig. 1). The complete amino acid sequence of Rabrab showed high amino acid identity to those of the rab1 (Table 1), which function in the vesicle transport from the ER to the Golgi complex. As shown in Fig. 1, the overall regions containing GTPase catalytic sites (I and II) and GTP/GDP binding sites (III and IV) were almost completely conserved, but there were differences in amino acid sequence at the carboxy-terminal region among all the living things listed.

Expression of cDNA for GST-Rabrab in E. coli and Purification of the Fusion Protein
cDNA for Rabrab was inserted into an expression vector and expressed in E. coli as GST fusion protein. The fusion protein recovered in the soluble fraction was purified with a glutathione S-Sepharose column for GST. SDS-PAGE analysis showed that the purified protein was homogeneous (Fig. 2). The evaluated molecular mass on SDS-PAGE was 49 kDa. About 1 mg protein was recovered from a 2-l culture of E. coli.
Fig. 2. SDS-PAGE Analysis of GST-BRab.
lane 1: molecular weight markers, and lane 2: GST-BRab (1 μg).

Fig. 3. Magnesium Dependence of GST-BRab on [35S]-GTPγS Binding.
GST-BRab was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. A reaction mixture (100 μl) containing 2.4 μg GST-BRab, 50 mM Tris-HCl (pH 8.0), 5 μM [35S]-GTPγS and respective concentrations of MgCl₂ was incubated at 30°C for 120 min. Thirty-six p m mole of bound [3H]-GTP without MgCl₂ was defined as 100%.

Characterization of GST-BRab
i) GTPγS and GDP Bindings. From the course of GTPγS binding, equilibrium conditions of bindings were almost reached after 120 min. To examine the effects of magnesium ion on [35S]-GTPγS binding, GST-BRab was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, to remove guanine nucleotide, and then the binding activities to [35S]-GTPγS at varying concentrations of MgCl₂ were measured (Fig. 3). Optimal binding of [35S]-GTPγS was observed at 6 mM MgCl₂, where the binding was increased 25% compared with that without MgCl₂. The binding activities of purified GST-BRab to [35S]-GTPγS and [3H]-GDP in the presence of 6 mM MgCl₂ were measured. From Scatchard plots of the bindings (Fig. 4), the values of Kₐ (association constant) of GST-BRab for [35S]-GTPγS and [3H]-GDP were calculated to be 1.5 × 10⁹ and 0.58 × 10⁹, respectively. GST-BRab could apparently bind 0.9 mole of GTPγS and GDP. We examined competitive inhibition of [35S]-GTPγS binding to GST-BRab with various nucleotides (Table 2). The binding did not compete with ATP, TTP, CTP, and UTP (less than 10% inhibition was observed), but GTP, GDP, and GTPγS were very efficient competitors. This result indicated that GST-BRab specifically bound GTP and GDP, like the other small GTP binding proteins.

ii) GTP Hydrolysis with GST-BRab. GST-BRab (2.4 μg) was incubated at 30°C for the indicated period of time in 25 μl of reaction mixture [50 mM Tris-HCl (pH 7.8), 1 mM DTT, 20 μM [3H]-GTP and 6 mM MgCl₂ or without MgCl₂]. One hundred μl of 0.5 M EDTA was added to separate guanine nucleotide from [3H]-GTP-bound and [3H]-GDP-bound GST-BRab. [3H]-GDP was separated on a TLC plate (PEI-C) and its radioactivity was counted. The radioactivity around the origin was

Table 2. Competition of GTPγS Binding to GST-BRab with Nucleotides.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>GTP</th>
<th>GDP</th>
<th>GTPγS</th>
<th>ATP</th>
<th>TTP</th>
<th>CTP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition (%)</td>
<td>100</td>
<td>92</td>
<td>97</td>
<td>1.1</td>
<td>0.0</td>
<td>4.3</td>
<td>8.6</td>
</tr>
</tbody>
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Fig. 4. Scatchard Plots of GTPγS (▲) and GDP (■) Bindings to GST-BRab.
A reaction mixture (100 μl) containing 2.4 μg GST-BRab (50 pmole), 50 mM Tris-HCl (pH 8.0), 6 mM MgCl₂ and respective concentrations of either [35S]-GTPγS or [3H]-GDP was incubated at 30°C for 120 min.
Expression and Properties of BRab

Fig. 5. Course of GTP Hydrolysis with GST-BRab Activity was assayed at 30°C in 25 μl of a medium containing 1 mM DTT, 20 μM [3H]GTP, 2.4 μg GST-BRab, 50 mM Tris-HCl (pH 7.8) and 6 mM MgCl₂ (•) or without MgCl₂ (●). [3H]-GDP was separated on a TLC-plate (PEI-C) and its radioactivity was counted.

Fig. 6. Exchange of Bound [35S]-GTPγS or [3H]-GDP with GTPγS and GDP.

A reaction mixture containing 2.4 μg of GST-BRab, 50 mM Tris-HCl (pH 8.0), 6 mM MgCl₂ and either [35S]-GTPγS or [3H]-GDP was incubated at 30°C. The dissociation of either [35S]-GTPγS or [3H]-GDP from GST-BRab was started by adding a 1000-fold excess of either unlabelled GTPγS (●; for [35S]-GTPγS, ×; for [3H]-GDP) or GDP (◊; for [35S]-GTPγS, ▲; for [3H]-GDP). At the indicated period of time, the reaction was stopped and [35S]-GTPγS or [3H]-GDP bound to GST-BRab was measured as described in “Materials and Methods”. The amounts of [35S]-GTPγS (41.5 p moles) and [3H]-GDP (42.5 p moles) bound to GST-BRab at 0 min were defined as 100%, respectively.

not detected (100 cpm , data not shown). This result suggests that guanine nucleotide was separated from [3H]-GTP-bound and [3H]-GDP-bound GST-BRab with EDTA. Hydrolysis reactions with and without MgCl₂ proceeded linearly for at least 180 min (Fig. 5). The rates of GTP hydrolysis were 5 mol/min with 6 mm MgCl₂ and 4 mol/min without MgCl₂ per mole of BRab.

The Exchange of [35S]-GTPγS and [3H]-GDP.

The kinetics of exchange of [35S]-GTPγS and [3H]-GDP on GST-BRab were examined (Fig. 6). The rate constants of [35S]-GTPγS with GTPγS and GDP were 4.6 mol/min and 3.0 mol/min, and those of [3H]-GDP with GTPγS and GDP 8.9 mol/min and 7.7 mol/min per mole of BRab, respectively.

Discussion

One cDNA clone encoding a small GTP binding protein (BRab) was isolated from a brain cDNA library of Bombyx mori. It encodes a polypeptide with 202 amino acids sharing 60–80% similarity with rab1 family members (Table 1). The regions relating to GTPase catalytic sites (I and II) and GTP/GDP binding sites (III and IV) were almost all conserved. These results identified BRab as one of the small GTP binding proteins. The carboxy-terminal region of small GTP binding protein has been suggested to be important for its function.16-18 For example, cysteinyl residues in this region are modified with fatty acids, contributing to binding of BRab to biological membrane.19-21 Further this region may function as the site interacting with other regulatory proteins such as GTPase-activating protein (GAP), guanine nucleotide exchange protein (GEP), and GDP dissociation inhibitor (GDI).22-24 The diversity in the amino acid sequences of carboxy-terminal regions of insect small GTP binding proteins may be necessary as a functional portion to interact with regulatory protein, resulting in the stimulation of neuropeptide release in insect brain.

To characterize the biochemical properties, cDNA for BRab was expressed in E. coli as a glutathione S-transferase fusion protein (GST-BRab). The recombinant protein recovered in the soluble fraction was purified to homogeneity with glutathione Sepharose (Fig. 2). First, the magnesium dependence of GST-BRab on [35S]-GTPγS binding was examined. Optimal binding was observed at a concentration of 6 mM of MgCl₂ (Fig. 3). The purified GST-BRab could bind GTPγS and GDP with association constants (Kₐ) of 1.5 × 10⁶ M⁻¹ and 0.58 × 10⁶ M⁻¹ in the presence of 6 mM MgCl₂ (Fig. 4). In competition of [35S]-GTPγS binding with various nucleotides, it was indicated that BRab bound specifically to GTP, GDP, and GTPγS (Table 2). Although GST-BRab bound weakly to GTP, compared to the other small GTP binding proteins, with Kₐ values, Ras: 33 × 10⁶ M⁻¹,25 Rab3A: 22 × 10⁶ M⁻¹,26 and RhO A: 33 × 10⁶ M⁻¹,27 it had a higher affinity with GTPγS than GDP.

In general, the GDP-bound state of small GTP binding protein is the inactive form, while the GTP-bound state is active form.1-2 Therefore, GTP hydrolysis and replacing GDP with GTP are essential steps in the functional cycle of small GTP binding protein. GST-BRab had GTP hydrolyzing activity, 5 mol/min/mole of BRab in the presence of 6 mM MgCl₂ (Fig. 5). As shown in Fig. 6, the rate constants for exchange of [35S]-GTPγS with GTPγS and GDP were 4.6 mol/min and 3.0 mol/min, and those of [3H]-GDP with
GTPγS and GDP 8.9 m mole/min and 7.7 m mole/min per mole of BRab, respectively, indicating that GTPγS exchanged [3H]-GDP from GST-BRab most efficiently among all listed. These results suggest that the GTP-bound BRab converts into the GDP-bound state by intrinsic GTP hydrolysis activity, and the GDP-bound BRab returns to the GTP-bound state with the exchange of GDP with GTP.

It is accepted that rab1 regulates the transcytosis from one membrane to the other, as observed in the vesicle transport from the ER to the Golgi complex, where calcium ion also plays an important role. Therefore, both calcium ion and rab1 are required for the fusion of carrier vesicles with the membrane of Golgi complex. In the insect brain, calcium ion is important in neuropeptide secretion. The increase in concentration of intracellular calcium ion with calcium ionophore in the brain-cortex cardium-corpus allatum (Br-CCA) of B. mori was found to be essential for the carbachol-induced PTTH release. Further approximately 25-kDa and 21-kDa proteins in Br-CCA were specifically phosphorylated with the treatment of carbachol via Ca-regulated protein, like calcium/calmodulin dependent protein kinase. In addition to calcium ion, this BRab may also be required for controlling PTTH secretion from neurosecretory cells in the brain of B. mori. BRab may be modified by Ca-regulated protein, causing the change in interaction with regulatory proteins such as GEP, GDI, and GAP, and eventually regulate the release of contents of secretory vesicles from the axon end of neurosecretory cells. Since little is known about the function of regulatory proteins in signal transduction in insect neurosecretory cells, further investigations are in progress to explore these regulatory proteins and their functions on BRab.

References

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